DESCRIPTION OF THE INVENTION

RETROVIRAL RECOMBINATION ASSAYS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to United States Provisional Patent Application Serial No. 60/143,015, entitled "Measuring Genetic Recombination in HIV", filed July 9, 1999, and to United States Provisional Patent Application Serial No. 60/164, 626, entitled "Translentiviral Vectors and Transduction of Eukaryotic Cells Therewith", filed November 10, 1999, each of which applications is herein incorporated by reference in its entirety including all drawings.

BACKGROUND OF THE INVENTION

The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art, or relevant, to the presently claimed inventions, or that any publication specifically or implicitly referenced is prior art.

This application relates to retroviral gene therapy vector design, safety, and quality assurance.

Retroviruses are characterized by a unique replication strategy in which the genome is RNA, and is reverse transcribed into a linear double-stranded DNA, which is subsequently integrated into a host cell genome. From this "provirus" is spawned copies of the retroviral genomic RNA for encapsidation into virion particles that are then exported out of the cell to start the viral life cycle anew. The virion particles are manufactured in the cell according to genetic specifications encoded within the proviral genome.

All known retroviral genomes contain three major coding domains that direct virion production and replication: gag, which directs the synthesis of structural proteins that form the matrix, the capsid, and the nucleoprotein structures; pol, which contains the information for the reverse transcriptase and integrase enzymes; and env, from which are

derived the surface and transmembrane components of the viral envelope glycoprotein. An additional smaller coding domain present in all retroviruses is pro, which encodes a protease enzyme that is recruited to the inside of the virion to effect maturation of fusion polyprotein products therein. The lentivirus subfamily of retroviruses further encodes pro, which is a part of pol (PR, RT, IN).

Retroviruses may generally be divided into two classes: simple and complex, depending on the organization and representation of the genome. Complex retroviruses, in contrast to simple retroviruses, encode additional accessory and regulatory proteins that may be derived from differentially spliced messages coming from the genome. Complex retroviruses may further be divided into additional subgroups, among which are the lentiviruses.

In nature, lentiviruses are known to cause disease, principally by killing or inducing loss of function of specific cells and tissues. One of the most well characterized lentiviruses is HIV, the causative agent of Acquired Immuno Deficiency Syndrome (AIDS) in humans. A comprehensive description of different retroviruses, their biology, and genetic organization may be found in Coffin et al., eds., *Retroviruses*, Cold Spring Harbor Laboratory Press, New York (1997), of which the skilled artisan is aware.

Man-made retroviral vectors including HIV-based lentiviral vectors exist and offer significant utility in being able to transduce a variety of nondividing tissue cells and sustain expression of transgenes in vivo. Akkina et al. (1996) J. Virol. 70: 2581-2585; Naldini et al. (1996) Science 272:263-267. Such tissues include but are not limited to brain, liver, muscle, and hematopoietic stem cells. Lentiviral vectors in particular hold great promise for gene therapy, and clinical trials to evaluate their safety and efficacy for treating certain human disease are being considered.

However, despite the advances and the promise these vectors hold, safety concerns still exist. Replication incompetent retroviral vectors can potentially recombine to form replication competent retroviruses (RCRs). This possibility, especially in the instance of pathogenic lentiviruses, should be minimized to maximize safety. To date, lentiviral vector systems have partially addressed this issue by expressing the essential viral genes on separate genetic elements (so-called "split-function") vectors. Naldini et al., supra;

Kafri et al. (1999) J. Virol. 73:576-84; Wu et al. (1997) EMBO, vol. 16, no. 16, pp. 5113-5122. Nevertheless, the possibility still exists that RCRs can form from these split function systems, and current in vitro cell culture and animal model systems are limited in their ability to monitor and evaluate this.

A retroviral assay system that can sensitively monitor genetic recombination events is therefore needed.

SUMMARY OF THE INVENTION

The invention features methods, systems, and indicator cells that provide for a sensitive determination of retroviral recombination and/or the potential for production of RCR or the emergence in vivo of potentially pathogenic viral forms.

The recombinants can contain one or more retroviral genetic determinants, e.g., gag, pol, env, tat, and /or rev, which can originate from one or more packaging constructs such as is generally used in split function retroviral packaging systems. Env is usually supplied in its own construct. Nucleic acids that encode the packaging component of the vector particles are generally not efficiently packaged into retroviral virion. The principal function of the packaging construct(s) is to package/encapsidate and help transfer a gene-transfer construct. These packaging constructs supply *in trans* the necessary structural and enzymatic retroviral proteins. "SIN" vectors are self-inactivating vectors that have, for example, deleted U3 sequence in the 3' LTR. Upon one round of replication, these changes are copied into both the 5' and 3' LTRs, thereby producing a less transcriptionally active provirus.

The invention can measure recombination events that unite one or more of the necessary genetic determinants contained within a wide variety of packaging and gene-transfer constructs. The result is detected by the establishment of a new, "recombinant" structure that contains genetic elements or portions thereof that were formerly found separately in the packaging and gene transfer construct components of the system. It is possible, although less likely so in the instance of SIN vector use, that these recombinant nucleic acids can then be packaged and mobilized to other cells because the recombinant

nucleic acids contain, and thereby may encode, at least some of the necessary components for replication and mobilization.

Because the functional retroviral gag-pol genetic structure is required for any type of RCR or viral DNA mobilization, monitoring for its presence in the form of a retroviral genetic recombinant allows a means to assess the risk of a retroviral/lentiviral vector creating an RCR event. As such, the invention provides a basis to measure the safety of vector stocks for use in gene transfer by using gag and pol, and more specifically gag-pol, as a surrogate marker for RCR.

Thus, in a first aspect the invention features a method of detecting a retroviral genetic recombinant, preferably a recombinant having gag and pol functions which can be complemented by helper function(s) supplied by or to the cell, and which helper function(s) facilitate propagation of the recombinant to permit the recombinant's detection. This detection can take place using any one of a variety of biochemical, diagnostic, and/or functional assays that can identify the recombinant. Preferably, the assay makes used of the gag and pol functions contained within the recombinant, and a marker gene to facilitate the detection.

By "gag and pol functions" is meant to either a gag-pol fusion arrangement or to the supply of the individual gag and pol elements separated from one another. Some retroviruses, e.g., lentiviruses are characterized by the former, and others, e.g., spumaviruses, by the latter. By "gag function" is meant a viral packaging function sufficient to produce a virion or virus-like-particle that is capable of encapsidating a retroviral genome. Such gag function does not necessitate expression of the entire gag sequence (Accola and Gottlinger (2000) J. Virol. 74:5395). By "pol function" is meant any one of the activities (enzymatic or non-enzymatic) or combinations of activities provided by protease (PR), reverse transcriptase (RT), and integrase (IN). These functions may be provided by the expression of the entire pol gene (PR, RT, IN), or by separate expression of the individual components thereof.

By "retroviral genetic recombinant" (hereinafter "recombinant") is meant a species of retrovirus that has undergone a nucleic acid recombination event, e.g., between one or more nucleic acid strands or constructs, e.g., packaging and vector constructs of split

function retroviral systems. The retroviral genetic recombinants of the invention may, in addition to, and as a consequence of being derived from the events described above, also contain man-made or artificial sequences, including heterologous sequences such as reporter/marker gene sequences and mutations introduced into coding and non-coding regions.

"Propagating" or "propagated" refers to the ability of the recombinant to integrate and duplicate in the host cell, e.g., as part of a mitosis event, and/or to support viral replication and mobilization, e.g., using various helper functions that are supplied by or to the host cell. "Helper functions" are used to help facilitate propagation of the recombinant when the recombinant is one that is incapable or inefficient at replicating, packaging, and/or infecting by itself Examples of helper functions include but are not limited to the retroviral env gene product and pseudotypes thereof, and/or the retroviral tat and rev gene products. Depending on the embodiment, various helper functions can be provided individually or in combination. Usually, although not necessarily, these helper functions will originate from a different nucleic acid strand in the cell relative to the recombinant strand, although each may ultimately share the feature of being integrated into the host genome. This is usually a consequence of the "split function" aspect of most retroviral systems that is directed to "disarming" viruses and promoting biological containment and safety.

"Providing a cell suspected of having said recombinant" embraces any manner in which the introduction or establishment of such recombinant can occur, including but not limited to infection, transfection, transduction, and transformation, as those techniques are commonly understood. The recombinant may be formed in the cell itself, e.g., from different or related genetic elements present on different nucleic strands, at a step upstream to this, e.g., in another cell from which the intact recombinant is then transferred to the present cell, e.g., via mobilization, or formed in a virion particle. However, exactly how the recombinant is formed is not intended to be limiting of the invention.

The term "mobilization" is broad. It can relate to the packaging and/or export of a nucleic acid sequence, usually to another cell. The nucleic acid sequence is preferably either a retroviral genetic recombinant nucleic acid sequence or a marker gene flanked by

sequences that facilitate its mobilization and expression, e.g., packaging into a retroviral-like particle, reverse transcription, integration, and promoter-specific expression. The packaged nucleic acid may be contained within a retroviral virion, virus like particle, or capsid structure, which may also be isolated and assayed, e.g., extracellularly as a viral supernatant. Usually, although not always, the packaging of the nucleic acid is facilitated by a viral packaging signal. For transfer/mobilization into another cell the nucleic acid sequence will also preferably contain cis-acting signals that facilitate reverse transcription and integration. If the mobilized nucleic acid is the marker gene, the flanking sequences will also preferably contain a promoter for the expression of the marker gene. The promoter may be an inducible promoter or a constitutive promoter. A preferred embodiment for the latter is the human phosphoglycerate kinase gene promoter, "pGK". See Michelson et al. (1985) PNAS 82:20, pp. 6965-6969 and GenBank accession M11958.

By "determining the presence of said retroviral genetic recombinant" can mean any assay that can detect mobilization or a recombinant, including but not limited to e.g., hybridization assays, i.e., fluorescent in situ hybridization (FISH), antigen-detection (e.g., ELISA), polymerase chain reaction (PCR), genetic marker response/mobilization, and/or recombinant mobilization. One or more such assays can be used together or independently in making the determination. The features of the individual cells used in the method, and at each step (where multiple steps requiring multiple, sometimes different, cells are undertaken), may vary depending on the precise embodiment. An example is illustrated by use of the claim term "optionally" which is meant to denote that various embodiments are possible, some which utilize (and therefore require) a marker gene present in the cell, and those that do not require such a marker gene's presence. An example of the latter is when the assay relies solely on antigen-detection, PCR, or marker or gene mobilization to a second cell that may or may not contain such a marker.

The individual embodiments noted are not necessarily mutually exclusive, and may be combined in certain other embodiments. Thus, in some embodiments both the marker gene and one or more other ways of determining the presence of the recombinant are used, e.g., PCR and antigen-detection. In other preferred embodiments the marker is used and

must be present in the recited cell, e.g., to denote the presence of and/or enrich for recombinants from among a greater population of non-recombinants, e.g., in a viral stock.

In preferred embodiments, the retroviral genetic recombinant is integrated into a cellular chromosome and propagated or capable of propagation along with the cell, e.g., as a provirus. The marker gene, if it is present in the cell, is preferably also integrated.

While the recombinant may harbor any one of a number of retroviral sequences, e.g., coding and/or cis-acting sequences, other sequences including heterologous sequences may also be included. Examples of retroviral coding sequences include gag, pro, rt, in, pol, gag-pol, gag-pro, tat, rev, env, vpr, vpu, and vif. "Cis-acting" sequences include those that possess functions in addition to or separate from coding sequences, e.g., promoters, enhancers, frame-shift signals, polyadenylation signals, primer binding recognition sites (PBRs), LTRs, portions thereof, etc. Heterologous sequences may include reporter genes and inducer or repressor molecules, e.g., that can activate or repress a marker gene.

Preferably, the recombinant contains at least a gag sequence and at least some pol sequence, or derivative sequences thereof. Derivative sequences may include mutations consisting of silent mutations, stop codons, deletions, and/or insertions. The recombinant may also consist, at least in part, of a synthetic sequence, e.g., one having silent mutations in one or more codons, or with stronger or weaker codon usage in the target host than the natural retroviral coding sequence. By "gag and pol functions" is not necessarily meant "functional gag-pol". The latter term denotes coding sequence function and activity whereas the former can denote inactive presence, e.g., when in mutated format or when otherwise incapable of being propagated in the absence of "functional gag-pol" or functional gag and functional pol.

In embodiments wherein a mobilization is used to determine the presence of the recombinant, that mobilization may be of the recombinant itself and/or of a marker gene. For this, the marker gene preferably has a retroviral packaging sequence, a promter, and flanking LTR elements, like the recombinant. The promoter may be an inducible promoter or a constitutive promoter.

Mobilization may be fostered by the in trans supply of an env gene or pseudotype thereof. The env gene may have previously been introduced to the cell, but preferably is introduced immediately prior to a desired mobilization event, e.g., in the preferred form of a pseudotyping agent such as a VSV-G protein, however introduced. "Pseudotyping" is a commonly understood term of art used to denote the combining of heterologous envelope species and/or other retroviral components, which may allow for modified function over the wild-type retrovirus, e.g, the ability to infect or replicate in a broader or narrower host cell range.

In especially preferred embodiments, the marker gene is a selectable marker gene, preferably one that encodes antibiotic resistance, preferably resistance to an antibiotic, and most preferably resistance to the antibiotic puromycin. In other aspects and embodiments, the marker gene can be synonymous with a reporter gene, e.g., luciferase, green fluorescent protein (GFP), or B-galactosidase. Preferably the marker gene is affixed to a promoter, inducible or constitutive, that is used to drive expression of the gene within a host cell.

In a second aspect, the invention features a method for detecting a retroviral genetic recombinant having gag and pol functions. The method includes providing the recombinant in a cell as described for the first aspect, and additionally includes a marker gene in the cell that can be mobilized in the presence of the recombinant and one or more helper functions as described previously to thereby facilitate detection of the recombinant.

In one preferred embodiment, the marker gene and recombinant are integrated into the host genome, with each capable of expressing a gene. Preferably, the gene expressed by either, including the recombinant, may be detected using an assay. The assay for detecting the recombinant's expressed gene can include, but is not limited, to FISH, PCR, antigen detection, Tat transfer, Gag transfer, and nucleic acid or gene mobilization.

In a preferred embodiment, the recombinant includes one or more retroviral coding and cis-acting sequences, with the coding sequences preferably encoding one or more members selected from the group: gag, pro, pol, gag-pol, gag-pro, and derivatives thereof. Derivatives may include mutations such as insertions, deletions, silent mutations, and stop codon introduction.

In other embodiments, the recombinant has genetic elements that are used to mobilize itself and/or another nucleic acid sequence, e.g., a marker gene provided in the host cell.

Preferably, helper functions as described above are used to help facilitate propagation of the recombinant. These can include, for example, the retroviral env gene product and pseudotypes thereof, and/or the retroviral tat and rev genes. Thus, various helper functions can also be provided in combination, e.g., by providing each of env, tat, and rev where appropriate.

In preferred embodiments, the marker is, once again, integrated into the host cell genome, e.g, into one or more of its chromosomes. For this, the host cell is assumed to be a eukaryotic host cell, preferably a mammalian cell, and preferably one capable of being infected or otherwise supporting replication of the subject recombinant. This may require helper functions.

Preferably the marker gene is a selective marker gene, more preferably an antibiotic resistance gene, and most preferably a gene encoding resistance to the antibiotic puromycin. The gene is capable of expression from a constitutive or inducible promoter, which is preferably provided and operatively attached. The marker gene is further preferably flanked by cis-acting sequences that provide for its encapsidation, reverse transcription, integration, and/or expression.

In another aspect, the invention features a method for detecting a retroviral genetic recombinant in a cell that also includes a marker gene that is responsive to the presence of the recombinant within that cell. The method includes measuring the response of the marker gene to thereby detect the recombinant.

In preferred embodiments, the marker gene response is the result of a specific gene product encoded by the recombinant. This gene product may be a retroviral gene, e.g., tat or rev, or else one that encodes a heterologous inducer or repressor protein. In any event, the gene product influences marker gene expression, preferably through the presence of a corresponding cis-acting promoter and/or other response elements.

In preferred embodiments that may further build on one or more of the above embodiments, the marker gene is also a mobilizable marker gene, meaning that it can be mobilized.

The marker marker gene can be a reporter gene or a selective marker gene. Preferably it is a selective marker gene, more preferably an antibiotic resistance gene, and most preferably a puromycin resistance gene. The marker preferably has an expression that is controlled by a promoter that is present, whether inducible or constitutive.

In another aspect, the invention features a retroviral assay system for detecting a retroviral genetic recombinant having gag and pol functions. The system features a cell suspected of containing the recombinant, propagation of which is facilitated in the presence of one or more helper functions. The system further features a means for detecting the recombinant. The means includes, but is not limited to expressing a marker gene that is also present in the cell. Preferably, the marker gene is responsive to the presence of the recombinant, preferably to a gene product encoded therein. Additionally or alternatively, the means for detecting the recombinant can include mobilizing the marker gene, mobilizing the recombinant; and/or assaying for a product encoded or otherwise produced by the recombinant or said marker gene.

Preferably, the recombinant derives from a retroviral vector, and more preferably from a lentiviral vector, which is a preferred embodiment for all the aspects. The lentiviral-based vector may include, but is not limited to, HIV-1, SIV, HIV-2, FIV, EIAV, BIV, CAEV, and OVINE.

Preferably, as with the foregoing aspects, the one or more helper functions is further included to allow or help propagate the recombinant. The helper functions that can be used include, but are not limited to, env and pseudotypes thereof.

Preferably, the means for detecting the retroviral genetic recombinant is the expression of a marker gene that is responsive to the presence of the retroviral genetic recombinant. The means may further include detecting one or more genes expressed by the recombinant.

In preferred embodiments, the marker gene encodes a product that can be selected for under one or more environmental conditions. Preferably, the marker gene is an

antibiotic resistance gene, and more preferably one that encodes resistance against puromycin.

Preferred embodiments for the assay system include components that perform or assist in performing PCR, FISH, antigen-detection, Tat transfer, Gag transfer, and gene mobilization, or any other biochemical assay.

In another aspect, the invention features an indicator cell for indicating the presence of a retroviral recombinant, an RCR event, and/or a wild-type retrovirus. The cell has an integrated marker gene, preferably a selectable marker gene. Preferably the marker gene is responsive to the presence of one or more genetic elements encoded by the retrovirus, e.g., tat, or a heterologous inducer or repressor protein. The marker gene may also be capable of mobilization as described above, and to that end may include cis-acting elements suitable for accomplishing or facilitating such.

In some embodiments, the indicator cell may also include helper functions, e.g., env or a pseudotype thereof, when the retrovirus is one incapable of replicating, packaging, and/or infecting by itself. Such helper functions may be heterologously engineered into the cell. In another embodiment, tat is built into the cell to assist retroviral expression, replication, and/or packaging.

The indicator cell is preferably an immortalized mammalian cell, more preferably one that is capable of propagating an animal retrovirus, preferably a lentivirus, more preferably a human lentivirus, and most preferably a human HIV lentivirus. The indicator cell can be HeLa, 293T, a hematopoietic stem cell, or a derivative thereof. Most preferably, the cell is a 293T cell.

Preferably, the selectable marker gene of the indicator cell is an antibiotic resistance gene, preferably one that encodes a product that is resistant to puromycin.

In another aspect, the invention features a method, retroviral assay system, or indicator cell of any of the preceding aspects and embodiments that is further used to evaluate the risk of producing a replication-competent retrovirus from a retroviral vector. For this, the starting product may be a retroviral recombinant, e.g., one lacking one or more functional members selected from gag, pro, rt, int, env, tat, rev, and/or other gene(s). By complementing the lacking function in trans, one can test the relative risk of a vector

for achieving replication-competence, especially in vivo. In this aspect, the invention may further feature a method, system, or cell used for determining the risk of producing a replication-competent retrovirus (RCR) from a test vector particle. This aspect may include any one or more of the embodiments described previously.

In one preferred embodiment, the method, system, or cell includes providing a cell with a selectable marker gene having an expression that is activated or enhanced in response to a protein encoded by a recombinant. The selectable marker gene encodes a product which can be selected for in a growth media for said cell under a defined environmental condition. The method, system, or cell may also include features that facilitate contacting the cell with a test vector particle that is potentially capable of producing the recombinant in the cell and then selecting for the expression of the selectable marker gene in the environmental condition to thereby determine the risk of the test vector particle producing a replication competent retrovirus.

As used herein, a "retrovirus" is any ribonucleic acid sequence that may be reverse transcribed into double stranded DNA and subsequently integrated into a host cell genome. Although the invention in principle applies to all retroviruses, preferred retroviruses and vector systems for use with the invention include the lentiviruses, most preferably those selected from the group consisting of HIV-1, SIV, HIV-2, FIV, EIAV, BIV, CAEV, and OVINE. The genomes for these lentiviruses, including the locations of the cis and trans elements contained therein, are all well known and available in the art, e.g., as found in GenBank. Although especially preferred for the invention is the use of HIV-1 (see Table 1, below, for genetics), other systems may be similarly designed, and with similar effect. It is further anticipated that various designs of vector systems having heterologous coding and cis elements (combining elements from different retroviral species) may also be successfully employed using the various aspects of the invention, and implemented without undue experimentation.

By "replication-competent retrovirus" (RCR) is meant one that can replicate. Three genes: gag, pol, and env are thought to be essential to wild-type retrovirus replication. One form of an RCR would include env on a common nucleic acid sequence with gag and pol. In addition, multiple "cis-acting" (noncoding) elements are generally

thought to be necessary, e.g., long terminal repeat (LTR) sequences, a retroviral packaging signal (Ψ), a polyadenylation signal, a promoter or promoters for expressing the genes (usually within the 5' LTR sequence), a transfer RNA binding site (PBS), a polypurine tract (PPT) that facilitates initiation of first and second-strand DNA synthesis, a repeated R region that is required for transfer of DNA synthesis between templates; and short, partially inverted repeats that are located at the termini of the viral LTRs and that are required for integration. It is also possible that an RCR could be produced, especially through recombination in vivo, that does not contain env. Retrovirus particles can be pseudotyped with non-env protein that mediates infection. (See, e.g., Endres et al., "Targeting of HIV- and SIV-infected cells by CD4-chemokine receptor pseudotypes", Science. 278:1462-1464; Mebatsion et al. (1997) "A CXCR4/CD4 pseudotype rhabdovirus that selectively infects HIV-1 envelope protein-expressing cells", Cell. 90:841-847; Schnell et al. (1997) "Construction of a novel virus that targets HIV-1-infected cells and controls HIV-1 infection", Cell, 90:849-857.) It is conceivable that cellular ligand-receptor interactions could mediate infection/entry or retroviral particles. As used herein, regardless of whether the RCR contains env on a single strand, an RCR will be symbolically depicted as LTR-Ψ-gag-pol-env-LTR, although this is not limiting to the definition of RCR. When integrated into a host cell, this structure constitutes one form of a "provirus."

By "test vector particle" is preferably meant a retroviral virion, virus-like particle, or retroviral vector particle bearing a nucleic acid construct that is suspected of possessing, or having the potential to generate, a recombinant in an infected host cell. The test vector particle may be present as part of a larger heterogenous population, and may have a minority representation in that population. The test vector particle is preferably capable of infecting a host cell. In preferred embodiments, that cell harbors a selective marker gene. Preferably, if the test vector particle is able to form a recombinant nucleic acid species, that species is the product of genetic recombination involving one or more constructs, e.g., packaging and gene-transfer vector constructs. Preferably, the test vector particle reflects a potential recombination event between such constructs, and it is a purpose of the assay to test for this. It is not necessary that the packaging and vector constructs from which the

test vector particle derives have homologous sequences, e.g., gag coding sequences. In some embodiments, one or more mutations is present in the retroviral sequence of a recombinant, including but not limited to internal stop codons, silent mutations and/or deletions within the gag and gag-pol sequences of either of these constructs.

A "gene-transfer vector" or "gene-transfer construct" as used herein does not require gag, pol, and env, and need only contain the "cis" signals noted above, or equivalents thereof, for vector packaging, reverse transcription, and integration. Most or all of the sequence encoding viral Gag, Pol, and Env can be absent and replaced with other sequences, e.g., those encoding a gene of interest such as a reporter gene. A transfer vector can be illustrated symbolically as LTR-Ψ-seq-LTR.

By "recombinant provirus" is meant one that is produced by at least one genetic recombination event between one or more nucleic acid strands or constructs, e.g., packaging and vector constructs of split function retroviral systems. Although preferable, such a recombinant may not be integrated into a chromosome of a cell.

A "risk of producing a replication-competent retrovirus" occurs by any recombination event that produces a recombinant provirus. The preferred purpose of the assay is to measure the *possibility* for creation of LTR-Ψ-gag-pol-env-LTR (an RCR), and not necessarily its actual occurrence. Thus the assay is meant to serve, at least in part, as a means to assess the safety of administering a given vector (e.g., in gene therapy applications) to an animal, especially in the long term.

A "selectable marker gene" may be a reporter gene but is preferably a gene having an expressed product capable of affecting the growth characteristics of a cell under one or more environmental conditions. The expressed product can encode, for example, a product that endows resistance to an antibiotic. Examples include dhfr, which confers resistance to methotrexate (Miller et al. (1985) Mol. Cell. Biol. 5:431-437); neomycin/hygromycin phosphotransferase, which confers resistance to G418 and hygromycin (Palmer et al. (1987) Proc. Natl. Acad. Sci: 84:1055-1059; Yang et al. (1987) Mol Cell. Biol. 7:3923-3928); and, most preferredly for the invention, a gene that encodes a product that is resistant to puromycin or phleomycin (Morganstern and Land (1990) Nucleic Acids Res. 18:3587-3596). As an alternative to antibiotic resistance, the

expressed product may overcome a metabolic deficiency of the host. Examples include *hprt* (Miller et al (1983) Proc. Natl. Acad. Sci. 80:4709-4713) and thymidine kinase *(tk)* (Shimotohno and Temin (1981) Cell 26:67-77; Wei et al (1981) J. Virol. 39:935-944), which allow host cells that are otherwise deficient for the functional genes to grow in minimal media supplemented with, e.g., hypoxanthine and thymidine.

In preferred embodiments, the selectable marker gene is preferably integrated into the host cell genome, and is preferably flanked by one or more nucleic acid sequences not natural to the host cell it is in. Preferably the gene is flanked on its 5' end by a retroviral LTR sequence that may promote expression of the gene, and a retroviral packaging signal, e.g., Ψ. In most preferred embodiments, the gene is flanked on both sides by an LTR or SIN sequence that, under appropriate conditions, can be packaged, transferred to other host cells, and selected for under one or more environmental conditions.

In another preferred embodiment of the method, system, or cell, the selectable marker gene is under control of an LTR promoter that is responsive to the retroviral Tat protein. The term "*Tat*" refers to a retroviral protein encoded by the retroviral tat gene. Tat activates transcription from the retroviral LTR promoter by binding to a proximal sequence, "TAR", that is located within the 5' LTR (the "R" region).

The terms "mobilized" and "mobilizing" as used herein denote the process, method, or ability of replicating a recombinant and/or other provirus, e.g., one containing a marker gene, and transmitting it to another cell. This may require helper virus or transfected genes that encode and express these products to supply helper functions, e.g., packaging, virion production, and receptor binding capabilities (e.g., env).

In other preferred embodiments of the method, system, or cell, the recombinant provirus includes a gag sequence that encodes a Gag protein. A "*Gag*" protein is a retrovirus structural protein that is processed into several smaller proteins that form the virion matrix, the capsid, and the nucleoprotein structures of a retrovirus. Preferably, one can detect Gag using one or more of several techniques common in the art, e.g., as an additional measure of determining the risk of producing a replication-competent retrovirus (RCR) from said test vector particle. These techniques include the use of the polymerase

chain reaction (PCR), antibody detection, e.g., as in a p24 assay, and/or other biochemical methods. These procedures and techniques are well understood in the art.

In still other preferred embodiments of the method, system, or cell, the recombinant provirus includes a gag-pol sequence that can transcribe and translate into Gag, Pro, RT, and INT proteins. The term gag-pol refers to a continuous nucleic acid sequence that includes at least a portion of the gag, pro, rt, and int retroviral genetic loci, preferably in that order. By "functional gag-pol" is meant a nucleic acid sequence that encodes functional Gag, Pro, and Pol protein products which together can promote the packaging and mobilization of a retroviral and/or recombinant proviral nucleic acid sequence.

Preferably, a step is also incorporated in which the recombinant provirus' presence is determined using an inhibitor of reverse transcripase, e.g., Nevirapine.

In other preferred embodiments of the method, system, or cell, and included as an additional measure of determining the risk of producing a replication-competent retrovirus (RCR), is the step and/or machinery necessary for mobilizing the selectable marker gene to another cell in which the marker gene can also be selectably detected. This embodiment may also include the use of an inhibitor of reverse transcriptase such as Nevirapine to identify that expression is derived from a recombinant gag-pol provirus.

In other embodiments, the method, system, or cell detects env minus recombinant provirus. By "Env" or "env" is meant, respectively, the retroviral envelope glycoprotein and its corresponding genetic locus. Retroviral surface and transmembrane components derive from the envelope protein and are necessary for the production of infectious retroviral virion particles. An "env minus" provirus is one that lacks one or more of these capabilities.

In still other embodiments, the recombinant provirus protein that promotes expression of the selectable marker gene is a Rev protein that functions by virtue of its ability to shuttle retroviral response element (RRE)-containing retroviral transcripts from the nucleus to the cytoplasm of a host cell for suitable expression. Thus, in such embodiments, the marker gene includes an RRE element that can interact with the Rev protein.

In preferred embodiments, the test vector particle derives from a recombination event that occurs in a split function lentiviral packaging system, e.g., one that includes at least one packaging construct and a vector construct, as those constructs are known in the art. An "env construct" or pseudotyping equivalent may also be included. These constructs, as explained above, typically supply *in trans* one or more of the necessary gag, pol, and env retroviral genes whose products are necessary for gene transfer. The test vector particle of the method, system, or cell may be present as a product of an earlier transfection event which yields a heterogenous population of virion encapsidated retroviral sequences, of which the test vector particle, if it represents or is to produce a recombinant provirus in an indicator cell of the assay method, system, or cell, may represent but a minority. A "test vector particle" may contain one or more nucleic acid sequences that constitute, or upon infection are capable of producing, a recombinant retrovirus.

In preferred embodiments, the envelope construct may include a pseudotype envelope sequence, such as derived from the G protein of Vesicular Stomatavirus (VSV).

The packaging construct may further include a promoter and a retroviral nucleic acid sequence that encodes one or more products necessary for retroviral packaging. The promoter can promote expression of at least one of the retroviral gene products necessary for retroviral packaging, e.g., Gag, Pro, and Pol. The promoter can optionally be an inducible promoter, preferably selected from the group consisting of TK, RSV, CMV, and TRE promoters, which promoters are known and widely available in the art for use with the invention.

In some embodiments, the vector construct may further include a gene of interest, e.g., a reporter or marker gene, that can be used to monitor the relative success or absence of recombination in the assay. Preferred reporter genes are selected from the group consisting of green fluorescent protein (GFP), luciferase, and B-galactosidase (B-Gal). The reporter may express from a promoter other than an LTR promoter, and may be an "inducible" or "constitutive" promoter as such promoters are known in the art. Promoters for use in the vector construct may include the same promoters listed above as possibilities for the packaging construct, e.g., the TK, RSV, CMV, and TRE promoters

In especially preferred embodiments, the selectable marker gene encodes resistance to an antibiotic, e.g., puromycin., and the cell in which it is provided is preferably infectable with the test vector particle used, e.g., a lentiviral based test vector particle can infect a HeLa or 293T human cell, or a derivative thereof. Preferably the cell line used is an immortalized mammalian cell line. Preferably, the selection can occur within a period of time of two days less, and with a clear distinction of "positives" from "non-positives."

In another aspect, the invention features a marker rescue assay system for detecting and/or analyzing a recombinant. The system includes an "indicator cell" having a selectable marker gene whose expression is activated or enhanced in the presence of a recombinant provirus, and whose gene product can be specifically detected or selected for in a growth media for said cell under a defined environmental condition.

Preferred embodiments for this aspect may encompass any of those embodiments already mentioned, or combinations thereof. For example, the recombinant may be a functional gag-pol recombinant in which the Gag and Pol retroviral gene products are expressed and can be transcomplemented with Env to result in packaging and mobilization of the marker gene, which is preferably integrated in the genome of the indicator cell line. In embodiments where the selectable marker gene is also present, it too can be mobilized and selected for in the cell it is mobilized to.

In yet another aspect, the invention features an indicator cell as noted above. The cell preferably has a marker gene, preferably a selectable marker gene, operably linked to a promoter. The marker gene exhibits altered expression in the presence of one or more proteins supplied, e.g,. from a recombinant provirus. In preferred embodiments, the proteins are retroviral proteins selected from the group consisting of Tat and Rev. Preferably, the selectable marker gene is flanked by LTR sequences and can be mobilized from said cell in the presence of functional Gag and Pol gene products. Env or pseudotypes thereof may also be used to accomplish this.

In a preferred embodiment, the indicator cell line contains a stably integrated copy of the marker gene. The marker gene is preferably on a nucleic acid strand that contains a signal for packaging, i.e., a retroviral packaging signal.

In another preferred embodiment, the indicator cell line may contain nucleic acid sequences for the expression of an env gene. The expression of env can pseudotype gene products of the recombinant provirus that contact the indicator cell line allowing the recombinant provirus to mobilize to another cell. Mobilization can be to another env containing cell or to other cell types. Mobilized recombinants can be detected using a variety of methods as described herein, including detection of gag gene product, i.e., HIV-1 p24 core protein.

A common theme that forms the basis for preferred embodiments of the indicator cell systems of the invention is the generation of recombinants preferably containing gag-pol. Such recombinant contains the minimal requirements, minus env and/or another helper function, that is necessary for replication and/or packaging. Therefore, it is principally gag-pol that the majority of assays, systems, and indicator cells described herein are intended to detect. However, Tat transfer and other marker transfer assay aspects may not require this.

Features of the claimed indicator cells may further include any use, feature, or combination of uses of features recited for the methods and system aspects.

In still a further aspect, the invention features a Tat transfer assay in which an indicator cell of the type described above is used. The indicator cell includes a selectable marker gene that is expressed in response to a retroviral Tat protein encoded by a recombinant provirus, and wherein the selectable marker gene encodes a product, preferably a product endowing puromycin resistance, which can be detected or selected for in a growth media for the cell under a defined environmental condition, preferably in two days time or less.

In another aspect, the invention features a Gag transfer assay that includes an indicator cell having a selectable marker gene. The selectable marker gene is expressed in response to a retroviral protein encoded by a recombinant provirus. The recombinant provirus includes functional gag and pol genes that are capable of producing functional Gag and Pol proteins. The selectable marker gene encodes a product which can be selected for in a growth media for the cell under a defined environmental.

In yet another aspect, the invention features a marker mobilization assay that includes an indicator cell. The indicator cell contains a selectable marker gene that is expressed in response to a protein encoded by a recombinant provirus. The marker gene can be mobilized from the indicator cell to another cell in the presence of functional Gag, Pol, and Env retroviral gene products. The marker gene encodes a product which can be selected for in a growth media for the cell under a defined environmental condition.

Preferred embodiments for the Tat transfer, Gag transfer, and marker mobilization assay aspects may include any embodiment or combination of embodiments noted for any of the aspects that is consistent with a useful purpose. Further, the method,system, and indicator cell embodiments may combine any of the other aspect embodiments described herein as appropriate to a purpose and practice within the broad spirit of the invention.

Advantages of the invention include the ability to detect retroviral genetic recombinants in sensitive fashion, and the use of gag-pol as a surrogate marker for identifying RCR potential. The invention further affords improved safety and quality control in the design, production, and implementation of existing and future retroviral vector systems, preferably in prelude to the use of such vectors in gene therapy. The invention is especially well-suited in assaying for recombinant provirus produced from packaging constructs in which gag-pol has been disarmed, e.g., through the inclusion of mutations and/or deletions.

Other advantages, aspects, and embodiments will be apparent from the figures, the detailed description, and the claims.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows an exemplary retroviral genome (HIV-1) having genetic components that can be used in conjunction with the methods, assay systems, and indicator cells of the invention. See Table 1 for more details.

Fig. 2 shows an embodiment of a split function lentiviral vector assay system having a packaging construct (pTRE-gag-pol), a vector construct (pHR-CMV-GFP), and an env construct (pVSV-G; also known as pMD.G).

Fig. 3 shows how one aspect of the invention can significantly improve on existing Tat-transfer assays by utilizing a novel indicator cell line bearing an antibiotic resistance gene (e.g., puro) that is capable of conveniently enriching for (or "capturing") the desired LTR-tat-LTR and other recombinations. The tat gene product is only expressed in the system when introduced in proviral form and is capable of specifically activating the LTR-driven antibiotic resistance gene, which can then be detected through routine selection means.

Fig. 4 shows how embodiments of the inventions can be used in tat transfer, gag transfer, and marker rescue/DNA mobilization assays to enrich for and detect the occurrence of retroviral genetic recombinants.

Fig. 5 shows a tat transfer assay embodiment that makes use of the sensitivity and enrichment provided by the specific indicator cell line HeLa-Puro. The figure shows how the desired tat-containing recombinant can be specifically selected for using an internal antibiotic resistance marker.

Fig. 6 shows a PCR assay embodiment that may be performed to further verify the presence of a tat-containing recombinant.

Fig. 7 shows a gag transfer assay embodiment that makes use of the sensitivity and enrichment provided by the specific indicator cell line HeLa-Puro.

Fig. 8 shows the results of a p24 antigen assay embodiment for the invention that demonstrates gag transfer and expression.

Fig. 9 shows a marker-gene rescue/DNA mobilization assay that makes use of the sensitivity and enrichment provided by the specific indicator cell line HeLa-Puro. Also shown is the mobilization of the puro marker gene from the HeLa-puro cell into another cell.

Fig. 10 represents one prediction of how recombination between packaging and gene transfer constructs may occur to produce an LTR-Ψ-gag-pol-LTR event.

Fig. 11 shows a PCR assay embodiment that can be performed to verify the 5' recombination event that produces the LTR-Ψ-gag-pol-LTR event.

Fig. 12 illustrates a sequence confirmation that genetic recombination has occurred between the packaging and gene-transfer constructs. The sequence analysis also illustrates that the recombinants have an open gag-pol reading frame.

Fig. 13 shows a PCR assay embodiment that may be performed to verify the 3' recombination event that produces the LTR-Ψ-gag-pol-LTR event.

Fig. 14 shows sequence analysis of the 5' (Panel A) and 3' (Panel B) recombinant junctions of the recombinant retroviruses obtained and their corresponding vector and packaging construct constituents as depicted in Figs. 11 and 13. The figure also illustrates a sequence confirmation that genetic recombination has occurred between the packaging and gene-transfer constructs. The sequence analysis demonstrates recombinant between the 3' end of the vector construct and the polyA tract of the packaging construct.

Fig. 15 demonstrates validation of tat transfer via recombinant provirus using the non-nucleoside reverse transcriptase inhibitor Nevirapine.

Fig. 16 demonstrates validation of gag transfer via recombinant provirus using the non-nucleoside reverse transcriptase inhibitor Nevirapine.

Fig. 17 demonstrates validation of marker mobilization via recombinant provirus using the non-nucleoside reverse transcriptase inhibitor Nevirapine.

Fig. 18 shows another split function vector (a translenti system) embodiment that is incapable of transferring functional gag-pol by virtue of abrogating the translation of RT-IN sequence from the packaging construct. This essentially "disarms" the RT and IN products downstream of pro that are part of the same mRNA transcript and normally part of the same fusion protein prior to post-translational modification and maturation of the independent proteins.

Fig. 19 shows that the Trans-lentiviral vector cannot transfer gag in a gag-transfer assay Confirmation of this is provided when Vpr-RT-IN is supplied in trans.

Fig. 20 shows 5' junction sequence for recombinant proviruses, demonstrating the preservation of a stop codon at the beginning of the RT coding region for the translenti system, whereas the rt (pol) gene in the lentiviral–derived recombinants has an open reading frame (no stop codon).

Fig. 21 shows a third generation lentiviral vector system in which the tat and rev genes are supplied on a separate construct.

Fig. 22 indicates that genetic recombination has taken place between the vector and packaging construct, since the puro marker gene is mobilized from the 293T-LTR-puro cell to the HeLa-tat cell. Panel A shows the assay overview using the third generation vector system. Panel B shows the plated/selected colonies under different experimental conditions.

Fig. 23 illustrates a lentiviral vector packaging construction embodiment in which expression of Gag-PR-RT-IN is regulated using an Internal Ribosomal Entry Site (IRES). See Example 10.

Fig. 24 illustrates a trans-lentiviral vector split packaging system embodiment in which Gag and Gag-PR-RT-IN are expressed from separate coding sequences to mutate the highly conserved stem-loop structure in both Gag and Gag-PR-RT-IN. This does not have deleterious effects on the formation of infectious particles. See Example 10.

Fig. 25 illustrates an MLV vector packaging construction embodiment in which expression of Gag-PR-RT-IN is regulated using an Internal Ribosomal Entry Site (IRES). See Example 10.

Fig. 26 illustrates an MLV-based vector split packaging system embodiment in which Gag and Gag-PR-RT-IN are expressed from separate coding sequences to mutate the highly conserved stem-loop structure in both Gag and Gag-PR-RT-IN. This does not have deleterious effects on the formation of infectious particles. See Example 10.

Fig. 27a illustrates Vpr-RT-IN expression plasmids containing 1-18 kb of stuffer DNA, e.g., derived from lambda DNA ("Vpr-RT-IN/ST") for identifying which will still efficiently provide RT and IN function in trans. See Example 11.

Fig. 27b shows how to humanize Vpr, RT and IN genes by PCR.

Fig. 27c shows the codon usage of highly expressed human.

Fig. 28a-d shows various embodiments for splitting the structure of Gag/Gag-PR-RT-IN into two separate coding sequences: one that express Gag and another that expresses Gag-PR-RT-IN.

Fig. 29 illustrates an MLV-based transfer vector that contains a DNA flat sequence derived from HIV-1. To facilitate initiating the central strand synthesis, the PPT sequence derived from MLV is inserted upstream of the cPPT sequence.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The basic components of traditional retroviral-based gene delivery systems include packaging, vector, and envelope elements that are usually supplied on separate genetic constructs to promote safety ("split-function"; see, e.g., Figs. 2,18 and 21). The packaging construct traditionally encodes the structural (*gag*) and catalytic (*pol*) proteins that are necessary to generate an infectious particle. The vector construct typically contains the genetic material of interest that is to be transferred into the target cell, along with a packaging signal (Ψ), a promoter for gene expression, and cis-acting sequences necessary for reverse transcription and integration such as the long terminal repeats (LTRs) and a primer binding site (pbs). The envelope (*env*) component, also supplied on a separate construct, mediates recognition and entry of the virus into the target cell and is usually a heterologous env protein such as the G protein from vesicular stomatitis virus (VSV-G).

The retrovirus gag-pol structure is so highly conserved among retroviruses that it is likely required for any type of retroviral RCR and DNA mobilization event. Therefore, sensitive assays capable of detecting the de novo establishment or absence of this entity in vector-transduced cells would be useful. Such in vitro assays could help to evaluate retroviral/lentiviral vector safety and predict the risk associated with RCR and retrovirus-induced DNA mobilization in vivo. Such assays would be especially indicated prior to use in clinical trials with humans to ensure that vector stocks are free of the potential to generate gag-pol containing recombinants.

The Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA) has established assay guidelines for screening retroviral vector-producing cell lines and supernatant for the presence of RCR. However, these guidelines have been established only for the safety of MLV-based retroviral vectors, and include specific PCR and immunological assays to monitor for evidence of replication-competent MLV. Guidelines have not been established for the monitoring of lentiviral vectors.

Thus, there is a need to develop assays for measuring the safety of lentiviral vectors. When applying, developing, and evaluating these assays, it is important to keep in mind that the safety of lentiviral vectors, particularly vectors based on HIV, should be held to a higher stringency as compared to the current retroviral-based vectors.

Three in vitro methods for assessing HIV-based lentiviral vector recombination and RCR have already been developed. Naldini et al. (1996) Science 272:263-267; Kafri et al. (1999) J. Virol., vol. 73, pp. 576-84. These assays include: (1) *tat* transfer, which monitors for the presence of the HIV *tat* gene in the target cell; (2) *gag* transfer, based on measuring the HIV-1 capsid protein (p24Gag) in the medium of vector-transduced cells; and (3) Marker Rescue, which looks for the transfer of a marker gene from vector-transduced cells to other cells. Transfer of the *tat* and *gag* genes are used as markers to determine if recombination between the gag-pol packaging and gene transfer vector elements has occurred.

It was our hypothesis that the traditional assays used to monitor recombination/safety are not sufficiently sensitive, and therefore are limited for detecting and analyzing genetic recombination. Therefore, we developed methods to increase the sensitivity for detecting genetic recombination. Our approach takes advantage that any RCR will require the gag-pol structure, and that we have demonstrated that it is possible to disarm gag-pol by separating Gag-pro from RT-IN. We developed a cell line that through the expression of an inducible marker gene can indicate when a recombinant is formed and integrated into a host cell chromosome including those containing gag-pol. This markedly improves the sensitivity in detecting genetic recombinants and allowed us to enrich for the recombinants in order to conduct detailed genetic and biologic analyses. Detecting recombinants not only provides a marker for evaluating the safety of lentiviral-based gene therapy vectors, but also provides the means to understand the nature of the recombinants at the genetic level, and additionally can help in the design of new vectors.

In designing and implementing our new assay system, we focused on monitoring genetic recombination between the packaging and gene-transfer constructs that yields functional gag-pol (e.g., LTR-Ψ-gag-pol-LTR) in transduced cells. An RCR event by comparison would be expected to be more unlikely since it would require, in addition to

the recombination necessary to generate an LTR-Ψ-gag-pol-LTR, an additional recombination to include a functional env gene with result that an LTR-Ψ-gag-pol-env-LTR sequence is formed. The chances of generating an envelope-minus recombinant lentivirus would therefore be expected to be greater than the chances of generating an RCR event. Nevertheless, the former can be used as a surrogate predictive marker for the latter. Based on this predicate, we hypothesized that sensitive in vitro assay systems capable of directly detecting genetic recombination between the packaging construct and transfer vector in vector-transduced cells especially those containing gag-pol could be formulated to provide reliable endpoints (surrogate markers) for evaluating safety and predicting the emergence of RCR and retrovirus DNA mobilization in vivo.

Detecting the transfer of the tat, gag-pol and/or marker gene(s) is used to determine if recombination has occurred between the gene-transfer and packaging element vectors. Transfer of a marker gene from one cell to another requires that a functional gag/gag-pol recombinant structure (LTR-Ψ-gag-pol-LTR) be formed in the vector-transduced cells. This enables the production of retroviral particles capable of packaging either or both the recombinant lentivirus genome (LTR-Ψ-gag-pol-LTR) and the selective marker gene (e.g., as LTR-Ψ-marker-LTR). Such particles, if pseudotyped with VSV-G or other envs, are capable of mobilizing retroviral sequences, including those having marker and/or tat, to other cells where, in the case of the marker, it can be tracked (e.g., using selective conditions). The pseudotyped env may be provided in trans by using a cell line capable of expressing VSV-G or other env genes capable of pseudotyping the recombinant.

Example 1: Generation of HeLa-puro: A Tat-dependent Indicator Cell Line Capable of Capturing Lentiviral Vector Recombinants

To genetically analyze HIV-based lentiviral vector recombination, we developed a cell line which could be used to specifically select and enrich for recombinant genomes arising from recombination between the packaging and gene transfer vector components. In this "indicator" cell line, the puromycin N-acetyl transferase (*puro*) gene was integrated into a HeLa cell with its expression under control of the HIV-1 LTR. Because the HIV-1 LTR is trans-activated by the HIV-1 tat protein, *puro* expression within the cell is induced when the *tat* gene becomes integrated into the host genome and expressed. With *tat* being

encoded on the *gag-pol* packaging construct only, recombination between the vector and packaging elements would have to occur in order for *tat* to be transferred into the HeLa-LTR-puro cell. Expression of the *puro* gene would therefore lead to the detection and enrichment of only those cells that harbor the *tat* gene.

To construct the line, we used an HIV-based vector to deliver the *puro* gene under control of the HIV-1 LTR. The pHR-puro expression plasmid was constructed using commonly available starting materials by ligating a PCR amplified DNA fragment containing the puromycin gene (derived from pPUR; Clontech Laboratories) into the pHR-CMV-lacZ plasmid (Naldini et al. (1996) Science 272:263-267) at the ClaI/XhoI sites. The vector stock encoding the *puro* gene was generated by cotransfection of pTRE-gag-pol, pVSV-G (Naldini et al. (1996) Science 272:263-267), and pHR-*puro* into 293T cells (ATCC). Culture supernatant was harvested 60 hours post-transfection and used to transduce a HeLa cell line (ATCC).

Because tightly regulated expression of the *puro* gene is in part dependent on its site of integration within the HeLa cell genome, it was important to select a cell clone that not only had a high level of *puro* expression in the presence of *tat*, but also displayed a low level of background expression in the absence of *tat*. To select for candidate indicator cell lines, individual cell clones were expanded in culture and screened for *tat*-dependent expression of the *puro* gene. Each clone was infected with a pseudotyped HIV-1-derived vector encoding the *tat* gene. One cell clone, henceforth termed HeLa-LTR-puro, was selected because it was able to grow and form colonies in culture media containing relatively high levels of puromycin (>30 ug/ml) following infection with the HIV vector, but displayed low level background resistance to puromycin (<0.5 ug/ml) in the absence of infection.

Using routine methodologies, one of skill in the art can readily create equivalent, and perhaps even superior, cell lines. However, for convenience, the HeLa-LTR-puro line noted herein was deposited with an International Depository Authority (IDA) (American Type Culture Collection ("ATCC"; 10801 University Boulevard, Manassas, VA 20110-2209) on 30 June 2000 under conditions satisfying the Budapest Treaty for biological deposits in support of patent applications. (accession number to be assigned).

Example 2: Vector Constructions

A three component lentiviral vector system is shown in Fig. 4. The component vectors are constructed as follows: The pCMV-gag-pol packaging plasmid was constructed by inserting an EcoRI/BamHI DNA fragment of pSG3 (coordinates 258 to 8384) into the pcDNA3.1(+) plasmid (InVitrogen) under control of the CMV promoter. The recombinant plasmid was then modified by introducing a 39-base pair deletion in the packaging sequence (Ψ), and a 1357-base pair deletion in the envelope gene (coordinates 5827 to 7184). The pHR-CMV-GFP plasmid was constructed by ligating a PCR amplified DNA fragment containing the GFP gene (derived from pEGFP-C1, Clontech Laboratories) into the BamHI/XhoI sites of the pHR-CMV-LacZ plasmid. Also, a 150 bp sequence of DNA (coordinates 4327 to 4483) containing the central polypurine tract (PPT) and central terminal site (CTS) was PCR amplified from the HIV-1 pSG3 molecular clone and ligated into the unique ClaI site of pHR-CMV-GFP. The sequences in the vector construct illustrate the frameshift mutation that is used to introduce the premature stop codon in the gag gene. This was accomplished by the fill-in of a ClaI restriction enzyme site that is present at the 5' end of the gag gene. The vector contains only the first 360 base pairs of the HIV-1 gag coding sequence.

One of skill in the art can readily construct equivalent vectors using the genetic element delineations for the HIV-1 genome as reflected in Fig. 1 and Table 1 (based on NCBI Genbank Genome Accession Number AF033819; SEQ. ID. No. 1). A more detailed explanation of these components and their function may be found in Coffin et al., of which the skilled artisan is aware. Those of skill will appreciate that allelic variations can exist between different isolates. In construction of the actual vectors described herein, isolates as described in Li et al. (1992) J. Virol. 66:6587 and Ghosh et al. (1993) Virology 194:858 were used.

Table 1: Genetic Elements and Coordinates of Human HIV-1

Genetic Element	Coordinates
R:	(1-96)
U5:	(97-181)
PBS:	(182-199)
gag:	(336-1836)
pro:	(1637-2099)
pol:	(2102-4640)
vif:	(4587-5163)
vpr:	5105-5339)
tat:	(5377-5591, 7925-7968
rev:	(5516-5591, 7925-8197)
vpu:	(5608-5854)
env:	(5771-8339)
nef:	(8343-8710)
PPT:	(8615-8630)
U3:	(8631-9085)
R:	(9086-9181)

Example 3: Preparation and Titration of Lentiviral Vector Stocks

Lentiviral vector stocks were produced by transfecting 5 ug of the pCMV-gag-pol packaging plasmid, 2 ug of the pVSV-G expression plasmid (also known as pMD.G; Ory et al. (1996) PNAS 93:11400) and 5 ug of the gene transfer (vector construct) plasmid into subconfluent monolayer cultures of 293T cells by the calcium phosphate DNA precipitation method. Supernatants were harvested after 60 hrs, clarified by low speed centrifugation (1000g, 10 min), and filtered through 0.45-um pore-size filter. The vector particles were then subjected to ultracentrifugation using a Beckman SW-28 rotor (23,000 rpm, 90 min., 4°C) to concentrate vector particles. The pellets were resuspended in 1.0 ml of DMEM., aliquoted and frozen at -80°C.

To determine vector titers, supernatant stocks of 1.0, 0.2, 0.04, 0.008, 0.0016, and 0.00032 ul were used to infect cultures of HeLa cell, and GFP positive (green) cell colonies were counted two days later using a fluorescence microscope.

Example 4: Tat Transfer Assay

To improve on the traditional tat transfer assay, we developed the cell line of Example 1 that, through the expression of an inducible marker gene, can indicate when a recombinant is formed and integrated into the host cell chromosome (Figs 3-5). Because the HIV-1 LTR is trans-activated by the HIV-1 Tat protein, puro (puromycin N-acetyl transferase) expression is induced to confer resistance only if the cell becomes infected with a tat-containing recombinant that integrates and becomes expressed. Since the tat gene is present within the lentiviral packaging construct (linked to gag-pol), recombinants of the vector and packaging constructs would likely contain tat. Thus, these integrated recombinant lentiviruses (provirus) would express the Tat protein and transactivate the expression of the puro gene and confer resistance in puromycin-containing media. This novel tat transfer assay markedly improves the sensitivity for detecting genetic recombinants and importantly allows enrichment for the recombinants, making it possible for the first time to conduct detailed genetic and biologic analyses.

Utilizing our novel system we detected the 1000 resistant colonies derived from the 10e7 T.U. of lentiviral vector, indicating that the frequency of tat transfer is about 10^{-4} to 10^{-5} (Fig. 5). The assay was performed as follows: 10^7 i.u. of the lentiviral stock was used to infect $5x10^6$ HeLa-LTR-puro indicator cells (MOI=2). As a control, the indicator cells were mock infected under identical conditions. Two days after infection, cells were trypsinized, split into five 100-mm tissue culture plates and cultured in media containing puromycin (5 ug/ml). After nine days of selection in puromycin containing media, the cells were fixed in methanol and stained with crystal violet. As shown, puromycin resistant colonies formed from HeLa-puro cells transduced with the lentiviral vector stock (approximately 1000 colonies per 10^7 i.u.). In contrast, no colonies arose from the mock-infected culture. These results suggested that the *tat* gene was transferred into the HeLa-puro cell from the lentiviral stock.

Although unlikely, the above results cannot exclude the possibility that the tat gene was transferred via plasmid carried over from the production of the vector stocks in the culture supernatants. To test this, HeLa-puro cells were infected with supernatants from the 293T transduced cells in the presence of Nevirapine (NVP), an inhibitor specific to the HIV-1 RT. NVP would inhibit only HIV-RT-mediated Tat transfer - but not if it was transferred through plasmid DNA. In the presence of NVP (25 ug/ml), no puromycin-resistant colonies were detected (Fig.15). This result indicated that tat transfer was dependent on the HIV-1 RT, suggesting that the tat transfer was mediated by recombinant lentivirus. The presence of tat in the HeLa-puro cell's chromosomes was confirmed by PCR using primers specific to the first exon (219 bp) of the HIV-1 tat gene (Fig. 6).

Example 5: Gag Transfer Assay

Because recombination can occur whereby only tat is combined with the gene transfer vector construct, it was unclear from the *tat*-transfer results as to whether functional *gag* and *pol* genes were transferred as part of the recombinant retrovirus into the HeLa-puro indicator cells. To determine if recombinants containing functional *gag* and *pol* genes were transferred, we developed a method of enriching for only those recombinants that have open *gag* and *pol* reading frames (resulting from recombination between packaging and gene-transfer vectors). An overview of this approach for detecting and characterizing lentiviral vector recombination is depicted in Figs. 4 and 7. The assay includes two steps. First, 293T cells (ATCC, Virginia, USA) are infected with vector stocks. It is in these infected cells that recombinants are likely to be generated and stably integrated. Such recombinant genomes can be expressed and mobilize retroviral sequences, especially when helper functions are provided in trans, e.g., env. Second, a Tat-inducible antibiotic resistance marker gene (e.g., puro) in the HeLa-puro indicator cell line is used to capture recombinants which contain functional gag and pol genes. Utilizing puromycin selection, we are able to specifically select and enrich for cells containing recombinant retrovirus such as LTR-Ψ-Gag-Pol-Tat/Rev-LTR. While this assay design has been termed gag-transfer, the results also demonstrate retroviral DNA mobilization.

Specifics of the assay we performed were as follows: the lentiviral vector stock (10^7 i.u., MOI=2) was first used to infect 293T cells. Similar to the transduction of the

HeLa-puro cell line, recombinants should also integrate into the genomes of the 293T cells, and those recombinants that contain an open *gag* and *pol* reading frames would express viral Gag and Gag-Pol proteins, produce progeny particles, and package the recombinant RNA genome. After two days, the transduced 293T cells were transfected with the pMD.G expression plasmid in order to pseudotype any progeny vector particles that may be produced. To increase production of progeny particles, a *tat/rev*-encoding expression plasmid was also transfected. Three days following transfection, culture supernatant was harvested and concentrated by ultracentrifugation. The concentrated supernatant was now used to transduce the HeLa-puro indicator cell line. The transduced HeLa-puro cells were placed under puromycin selection as described above followed by fixation and staining of the colonies that may have formed. When VSV-G was supplied in trans, 390 puromycin-resistant colonies formed. (Fig. 7)

Thus, lentiviral vector-derived recombinants could transfer functional *gag* and *pol* genes into its target cell (293T). This result suggested that the retroviral recombinant genome can be mobilized from the transduced 293T cells to the HeLa-puro cells if an envelope was provided *in trans*. Again, no colonies formed when supernatants from mock-transduced 293T cells were used to infect the HeLa-puro cells.

Gag transfer was further confirmed by measuring the amount of HIV-1 capsid protein (p24 antigen) released into the supernatant of the puromycin-resistant HeLa-puro cells. Fig. 8 Method: The 390 colonies which formed following selection in the puromycin-containing media were trypsinized and replated into replica cultures. One half of the cells were transfected with a plasmid expressing tat and rev while the other half was mock transfected. Culture supernatants were collected 60 hours later and a small amount was aliquoted and frozen at -80^{o}C. Meanwhile, the remaining supernatant was concentrated by ultracentrifugation as described above and resuspended in 100 ul of DMEM. Both the unconcentrated and concentrated supernatants were analyzed for p24 antigen using an HIV-1 p24 antigen ELISA assay (CoulterInc.). Fig. 8 shows that up to 95 ng/ml of CA could be detected in the culture supernatant. Moreover, we also found a significant increase (up to 10 fold) in capsid protein if the puro resistant cells were transfected with the tat expression plasmid, indicating that Gag expression is linked with

the HIV LTR promoter (Fig. 7). This result demonstrated gag and gag-pol transfer and mobilization of the retroviral recombinant genome from the transduced 293T cells to the HeLa-LTR-puro cells.

In all, Fig. 8 demonstrates that gag was transferred and expressed in the vector-transduced cell. The increase in p24 production with co-transfection of tat suggested that the gag gene was linked to the HIV-1 LTR, and provided additional evidence that recombination occurred between the gag-pol packaging construct and the LTR-containing gene transfer vector. Our results further show that the recombinants generated by the lentiviral vector system mobilized both tat and gag and pol genes from the vector transduced cells (293T) into the puro-HeLa cell when the envelope (e.g., as pseudotype VSV-G protein) was provided in trans (Fig. 9). This result indicates that the lentiviral vector can regenerate recombinants (such as LTR-Ψ-gag-pol-tat/rev-LTR) in the vector-transduced cell, and that this envelope-deficient recombinant retrovirus can be mobilized into other cells when the envelope is provided in trans.

To confirm that Tat expression was a product of LTR expression and not of the packaging vector, the reverse transcription inhibitor Nevirapine (NVP) was used to identify the source of expression. The specific HIV-1 RT inhibitor NVP blocked the mobilization of the recombinants from the vector-transduced cell to the HeLa-puromycin cell, indicating that the retrovirus DNA mobilization is specifically mediated by HIV-1 gag/gag-pol (Fig. 16).

Finally, the high molecular weight DNA was extracted from the puro-resistant cells using a Wizard™genomic DNA purification kit (Promega, Madison, WI). DNA fragments of the 5' recombinant virus region (from U3 to gag, ~2 Kb)(Figs. 11, 13,) and 3' recombinant virus region (from Tat to U3;~2.5Kb)(Fig. 14, 15B,) were amplified by PCR, and the sequences contained therein determined.

The 5' region of the recombinant was amplified using sense primer homologous to a sequence located in the U3 region of the 5'-LTR (sequence: 5'-ccg gaa ttc tgg cta act agg gaa ccc act gc-3'; SEQ. ID NO. 2) with an antisense primer that hybridizes to a sequence located at the 3' end of the *gag* gene (sequence: 5'-cgc gga tcc tta ttg tga cga ggg gtc gct gcc-3'; SEQ ID NO. 3). Amplification of a 2054 bp DNA product (the predicted size of

the U3-*gag* fragment) can only occur if the vector and packaging elements have combined through genetic recombination. A PCR product of the predicted size was amplified from the lentiviral-derived puromycin resistant colonies. Both PCR amplified DNA fragments were cloned and sequenced. In 10 of 10 clones, sequence analysis of the 5' PCR fragment confirmed the presence of U3 R U5 (the 5' LTR), and the Ψ packaging signal (from the gene transfer vector) linked with the gag gene from the packaging construct (Fig. 12).

Sequence analysis of the 3' PCR DNA fragment also revealed a genetic linkage between the packaging construct and the 3' sequence of the gene transfer vector (Fig. 14). Interestingly, the recombinant genomes contained the poly A signal and the poly A tract derived from the packaging construct, indicating that recombination had occurred between the poly(A) tract of the packaging construct mRNA and the untranslated sequence immediately upstream of the 3' LTR of the vector during reverse transcription (Fig. 14B).

These results collectively demonstrate that the novel recombination-dependent retrovirus DNA mobilization (gag-pol transfer) assays can detect envelope-minus recombinant lentivirus that arise (derived from the packaging construct and transfer vector).

Example 6: Marker Mobilization Assay

One of the primary concerns associated with the formation of a recombinant containing a functional *gag-pol* is the potential of mobilizing DNA from one cell to another. Such mobilization would both perpetuate and augment the potential of RCR. Since the *puro*-containing RNA transcript in the HeLa-puro indicator cell contains the HIV-1 packaging signal, this RNA could be packaged into viral particles derived from the recombinant. Such particles, if pseudotyped with VSV-G, should be capable of mobilizing the *puro* gene into another cell.

In the recombinant gag-pol-mediated marker gene mobilization assay (see Figs. 9, 17), we tested this. Specifically, we tested whether the recombinant lentivirus (LTR-Ψ-gag-pol-LTR) could mobilize a marker gene (e.g., in LTR-Ψ-puromycin-LTR) from the vector-transduced cell to another cell. We pooled the puromycin-resistant colonies resulting from the *gag* transfer experiment and co-transfected them with the pMD.G and tat/rev expression plasmids. Supernatant was harvested and used to transduce HeLa-tat

cells Felber et al., J. Virol. 64:3734; Wu et al., J. Virol. 69: 3389, followed by selection of puromycin-resistant colonies as previously described.

It seemed likely that the recombinant retrovirus particles could package both recombinant lentiviral genomes (LTR-Ψ-gag-pol-LTR) and lentivirus vector genome containing a marker gene (such as LTR-Ψ-puromycin-LTR) (Fig. 9). Figs. 9 and 17 show that the puro gene was mobilized by the recombinant. This transfer was completely inhibited by NVP (Fig. 17), indicating that the marker gene mobilization was mediated by HIV-1-based retroviral recombinants (gag-pol).

Taken together, these results indicate that this novel cell-based recombination assay enables the detection of envelope-minus recombinant lentivirus derived from the genetic recombination between the packaging and vector elements of the lentiviral vector system. The recombinants containing the functional gag-pol structure in the vector-transduced cells can integrate into the host genome, express viral proteins (including Gag and Gag-Pol), produce retroviral particles, encapsidate viral RNA, and mobilize the viral genome to new target cells when a helper function, e.g, env, is provided in trans.

Example 7: Molecular Characterization of Recombinants

Assay recombinants can be predicted to have the structure indicated in Fig 10, which shows the daughter recombinant molecule (LTR-Ψ-*gag-pol-tat/rev*-LTR) and parts of the parent molecules from which is formed the recombinant.

To verify this, we selected for cells harboring a *tat*-containing recombinant genome and expanded these cells in culture to characterize the nature of the genetic element responsible for transfer of *tat* into the HeLa-puro cell. Chromosomal DNA from a cell pool was isolated using the Wizard™ genomic DNA purification kit (Promega). If recombination occurred between the vector and gag-pol packaging components, then we would predict that *tat* was transferred as part of an LTR-Ψ-*gag-pol-tat*-LTR recombinant genome. To confirm this, PCR was used as described in Example 5 to amplify the recombinant from genomic DNA derived from the pool of puromycin-resistant HeLa-puro cells. Again, amplification of a 2054 bp DNA product is only able to occur if the vector and packaging elements have combined through genetic recombination. This, in fact, was confirmed.

Primers specific only to the pTRE-gag-pol packaging plasmid were used to determine if the packaging plasmid DNA was contaminating the genomic DNA preparations. The sequence of the 5' primer which hybridizes to the leader region of the CMV intermediate early promoter is 5'-gac ctc cat aga aga cac cg-3' (SEQ ID. No. 4). PCR failed to amplify a 1703 bp product, demonstrating that plasmid DNA was not contributing to the amplification of the recombinant genome.

The 5' and 3' primers used to amplify the 5' (2054bp) PCR product also encoded EcoRI and BamHI restriction sites, respectively. This allowed us to clone the PCR product into pUC119 vector DNA and sequence the individual clones. (Figs. 12, 14A) Our sequence analysis confirmed that the vector and packaging constructs had joined through recombination. Also, it appears that the recombination events on the 5' end occurred through homologous sequences because of the sequence overlap between the vector and packaging components. Since only recombinants with open gag reading frames are able to be transferred from 293T cells to the HeLa-puro indicator cells all recombinants sequenced (10/10) contained an open gag reading frame.

The 3' end of the recombinant was confirmed by PCR using a sense primer specific to the 5' end of the *tat* gene (sequence: 5'-ccg gaa ttc atg gag cca gta gat cct aga c-3'; SEQ. ID. No. 5) and with an antisense primer that hybridizes to the R region located in the 3'-LTR (sequence: 5'-cgc gga tcc gca gtg ggt tcc cta gtt agcc-3'; SEQ. ID. No. 6). (Figs. 14, 15B, 24B) As shown, PCR products of various molecular weights were amplified, with a predominant PCR product approximating 2500 bp in length.

The 3' (2500 bp) PCR product was cloned into a pUC19 vector DNA and individual clones sequenced. (Fig. 14B) Interestingly, the junction between the 3'-LTR and the packaging construct contained a polyA sequence of various lengths. Further analysis of the sequence revealed that the polyA sequence was derived from the mRNA transcript of the pTRE-gag-pol packaging component. These results demonstrated that the polyA tract can be used to promote non-homologous recombination between the vector and packaging constructs.

Although it seemed unlikely, it was possible that the *tat* and *gag* genes were transferred without recominbation (pseudotransduction), via the pTRE-gag-pol packaging

plasmid DNA carried over from the production of the vector stock. To test for this possibility, infections of the HeLa-puro cells were performed in the presence of Nevirapine, a non-nucleoside reverse transcriptase inhibitor specific to the HIV-1 RT. (Figs. 15-17) Nevirapine would prevent only HIV-RT mediated transfer of *tat* and *gag* into the HeLa-puro cell but not if it is transferred via plasmid DNA. These figures show the results of the tat transfer, gag transfer, and marker rescue assays which were performed as described before except that the infection of HeLa-puro was carried out in the presence of 1 ug/ml Nevirapine (NVP). No puromycin-resistant colonies formed in any of the assays, indicating that these transfers were mediated by HIV-1 reverse transcriptase and not through plasmid DNA contamination.

Example 8: Assay Comparing Gag-Pro versus Gag-Pol Vector Systems

We previously described a trans-lentivirus vector system based on a gag-pro (not gag-pol) packaging construct that promotes a "disarming" of lentiviral recombinants by eliminating production of functional reverse transcriptase (RT) and integrase (IN) from the traditional packaging vector. See United States Patent Application Serial No. 09/460,548. These necessary products (RT and IN) are instead expressed on their own, separate nucleic acid vector fused, e.g., to vpr, a sequence that when expressed is capable of promoting packaging of the fusion protein. See Wu et al. (1997) EMBO, vol. 16, no. 16, pp. 5113-5122, and United States Patent Application Serial No. 09/460,548, filed Dec 12, 1999, each of which is herein incorporated by reference.

We tested our new assay with our elegant trans-lentivirus system to see if we could produce recombinant lentivirus containing the functional gag-pol structure.

First, we tested the trans-lentiviral vector in the tat transfer assay, which does not require functional gag-pol. 293T cells were transfected with the four DNA components of the trans-lentiviral system (Fig. 18), and three days later the culture supernatants were collected. The vector particles were concentrated by ultracentrifugation, resuspended in DMEM, and titered by limiting dilution infection of the cell line, as described above. After nine day of culture in medium containing puromycin, approximately 800 resistant colonies were enumerated using bright-field microscopy (Fig. 18). This result suggests

that the packaging construct of the trans-lentiviral vector can recombine with the gene transfer construct.

To determine whether the novel trans-lentiviral vector design would prevent the generation of a recombinant containing the functional gag-pol structure, vector stocks of the trans-lentiviral vector were also analyzed for gag-transfer/DNA mobilization. Exactly as described above for the lentiviral vector (Fig.16), 10e7 infectious units of trans-lentiviral vector particles were used to infect 293T cells. Culture supernatants were collected and used to infect 293T cells as described above. Three days later these culture supernatants were collected and processed exactly as described above for the lentiviral vector. This analysis demonstrated that the trans-lentiviral vector is unable to produce resistant colonies, indicating added safety over the lentiviral vector. To confirm that this was the result of the separation of RT-IN (or RT and IN) from gag-pol, the experiment was conducted in parallel (Fig. 19) with an experiment in which the Vpr-RT-IN fusion protein was provided in the 293T infected cells (via transfection of the vpr-RT-IN expression construct). Under these conditions, approximately 300 resistant colonies were produced (Fig. 20). These results suggest that while recombination between the vector and packaging components can occur, the separation of RT-IN from gag-pol prevents the mobilization of the recombinant genome. Therefore, this approach disarms the gag-pol structure and represents a method for improving the safety of lentiviral vector gene therapy. It is also notable that in combination with assays to monitor/measure recombination and /or DNA mobilization, the trans-lentiviral vector safety can be quality assured by in vitro testing.

In order to confirm that the puro resistant colonies that were produced by complementation of the vector particles with the Vpr-RT-IN fusion protein were indeed RT-IN deficient, the resistant cells were expanded in culture and the high molecular weight DNA was extracted for genetic analysis (Fig. 20). This analysis demonstrated that that the RT-IN coding region was defective since 5 of 5 recombinants that were analyzed contained a translational stop codon (TAA) at the first amino acid residue of RT, as introduced into the trans-lentiviral packaging construct originally. By comparison, 5 of 5 recombinants of the lentiviral vector contained an open RT-IN reading frame (Fig. 20).

Our biologic and molecular results confirmed that the novel cell-based assay could sensitively monitor the recombinant gag-pol in the vector-transduced cells.

Example 9: Third Generation (Tat-minus) Vector Systems

Third–generation lentiviral vector systems remove the tat and rev genes from the packaging construct. While tat is not needed at all, the rev gene is still required and is therefore supplied in trans from a separate genetic construct (Fig. 21). Theoretically, this reduces the possibility for an RCR event. To avoid the dependency of a recombination assay on the presence of tat as a part of the lentivirus trans-lentiviral vector packaging construct, we assayed tat minus (3rd generation) vector for DNA mobilization in a tat-independent manner.

Fig. 22 shows an overview of such an assay embodiment, as well as data demonstrating that the puro gene in such an assay can be transferred into HeLa Tat cells if VSV-G is supplied. This is the identical concept behind the marker rescue assay described above.

For assaying the 3rd generation vector we found that, instead of HeLa-LTR puro, the 293T-LTR puro cell line improved sensitivity. 293T-LTR puro is constructed in the same way as HeLa puro. The same puromycin resistance gene is used and positioned downstream of an HIV-1 LTR, and the genetic transcript from which it is expressed contains an HIV-1 packaging signal that allows encapsidation into an HIV particle. The line 293T-LTR-puro was selected for its high expression levels of the puro-containing transcript. The more puro transcript available, the better the chance of it being incorporated into a viral particle for transfer into a cell, and the more efficient the system is.

Using routine methodologies, one of skill in the art can readily create equivalent, and, perhaps, even superior cell lines to 293T-LTR puro. However, for convenience, this line was deposited with an International Depository Authority (IDA) (American Type Culture Collection ("ATCC"; 10801 University Boulevard, Manassas, VA 20110-2209) on 30 June 2000 under conditions satisfying the Budapest Treaty for biological deposits in support of patent applications. (accession number to be assigned).

The third generation (tat-minus) vector stock is used to infect the 293T-LTR puro cell. If one of the 293T-LTR puro cells harbors an LTR-Ψ-gag-pol-LTR recombinant, the recombinant can express the viral Gag and Gag-Pol proteins and form a viral particle which can encapsidate the mRNA of the LTR-Ψ-Δgag-RRE–puro-LTR construct. Fig. 23 depicts and verifies this. Although the number of recombinants (LTR-Ψ-gag-pol-LTR) is fewer (52/10e7), the assay is sensitive enough to detect them.

Example 10: A Vector System Including Regulation of the Expression Level of Gag-PR-RT-IN Using an Internal Ribosomal Entry Site

Using the cell-based genetic recombination assay described above, we demonstrated that a new lentivirus vector packaging construct designed by splitting gag-pol into two components (gag/gag-pro and Vpr-RT-IN) significantly improved the biosafety of the lentivirus vector by preventing the regeneration of a recombinant lentivirus containing functional gag-pol. Since the gag-pol structure is highly conserved among all retrovirus, this structure must exist in any type of replicating-competed retrovirus derived from genetic recombination. By disarming the highly conserved gag-pol structure, we can improve the biosafety of all retrovirus-based vectors, including simple retrovirus-based vectors (such as MLV) and lentivirus-based vectors such as HIV, SIV, FIV, EIAV, BIV, Visna, CAEV, and Ovine lentivirus).

In this example, we removed the ribosomal frameshifting site in the retrovirus-based vector packaging system, which is high conserved among all retroviruses and essential for retroviruses to maintain a well regulated ratio of Gag proteins to Gag-Pol proteins in infected cells for producing infectious particles. This design splits the expression of Gag/Gag-PR RT-IN into two separate coding sequences: one that expresses Gag and another that expresses the Gag-PR-RT-IN. Here, the production of a fixed ratio of Gag to Gag-PR-RT-IN is regulated by the internal ribosomal entry site (IRES) derived from the encephalomyocarditis virus (EMCV), rather than by the ribosomal frame-shift site. Since the translational initiation efficiencies of the different IRES mutants differs considerably, this allowed us to select different IRES mutants to regulate the production of Gag/Gag-PR-RT-IN at a fixed ratio, similar to the ratio of Gag/Gag-PR-RT-IN derived from the natural Gag/Gag-PR RT-IN structure. Importantly, this demonstrates that one

can design transvector packaging systems for all retrovirus-based vectors since the approach does not require virion-associated proteins (e.g., Vpr and Vpx).

A specific nucleotide motif, the slippery sequence, as well as a downstream mRNA secondary structure are essential for the production of the fixed ratio of Gag to Gag-PR-RT-IN (Fig. 23 for lentivirus vector; Fig. 25 for MLV vector). The disruption of the highly conserved stem-loop structure completely blocks the production of the fixed ratio of Gag to Gag-PR-RT-IN, which is essential to produce the infectious particles.

By separating the expression of Gag and Gag-PR-RT-IN into separate coding sequences, we are able to mutate the highly conserved stem-loop structure in both Gag and Gag-PR-RT-IN constructs without deleterious effects on the formation of infectious particles (Fig. 24 for a trans-lentiviral vector system; Fig. 26 for an MLV-based vector). Since the highly conserved element no longer exists in this system, safety is now improved at two different levels: one in that Gag and Gag-PR-RT-IN are split into two separate coding regions which should prevent the regeneration of recombinant retrovirus containing the functional Gag-Pol structure; and two in that the novel packaging system completely removes the ribosomal frameshifing site which is essential for all retrovirus to maintain the ratio of Gag and Gag-PR-RT-IN during the production of infectious particles. It is likely that without this highly conserved element, any type of recombinant retrovirus would be unable to produce the infectious particles and replicate.

Since we express the HIV-1 Gag and Gap-PR-RT-INT polyproteins in two separate mRNAs, and the expression of Gag-PR-RT-IN polyproteins is no longer dependent on the highly conserved stem-loop structure of mRNA, this allows us to completely humanize all of the codons in the mRNA of Gag and Gag-PR-RT-IN. Therefore, modified gag-PR-RT-IN genes would express high levels of protein to produce high titer vector.

Example 11: Preventing Regeneration of Recombinant Lentivirus Vector Containing Functional Gag-PR-RT-IN

In current translentiviral packaging systems, there are large homologous sequences between the trans-enzyme plasmid (Vpr-RT-IN) and the packaging construct (gag/gag pro). If both mRNAs (trans-enzyme plasmid and packaging construct) are nonspecifically copackaged into the same virus particle, it is possible to regenerate the gag-pol structure

through homologous genetic recombination. There are two approaches to preventing the regeneration of the gag-pol structure. First, controlling the incorporation of Vpr-RT-IN mRNA into vector particles will eliminate the possibility of regenerating gag-pol since the retrovirus genetic recombination occurs within the viral particle. Second, minimizing the homologous sequence between the trans-enzyme plasmid and packaging construct will reduce the probability of regenerating gag-pol through homologous recombination. To do this, one can modify the Vpr-RT-IN plasmid at the genetic level in at least one of the following ways:

(a). Eliminating incorporation of mRNA of Vpr-RT-IN into vector particles. Although there appears to be no lower limit to the size of virion-incorporated mRNA in virus particles, there does appear to be an upper limit. The genome of a typical replication-competent murine retrovirus is about 8.3 kb, whereas that of Rous sarcoma virus, which contains src sequences in addition to the normal complement of viral genes, is about 9.3 kb. The maximum size for a replication-competent spleen necrosis virus vector is similar, about 10 kb (Gelinas et al PNAS (1986)). In the case of HIV-1, the size of the virus genome is about 9.2 kb. It is believed that a size greater than 18 kb may eliminate any possibility of packaging into the virus particle, particularly without an mRNA packaging signal. To do this, one may construct the Vpr-RT-IN expression plasmid to also contain 1-18 kb of stuffer DNA, e.g., derived from lambda DNA ("Vpr-RT-IN/ST"). These plasmids transcribe greater than 4 kb of mRNAs to express the Vpr-RT-IN fusion protein. To identify which of the Vpr-RT IN/st plasmids still efficiently provide RT and IN function in trans, one can test the different constructs compared with the parent plasmid, Vpr-RT-IN. (Fig. 27a)

(b). Eliminating incorporation of mRNA of packaging construct into vector particles One can construct the Gag-PR or Gag-PR-RT-IN expression plasmids containing 1-18 kb of stuffer DNA as described for (a), generating Gag-Pro/ST or Gag-PR-RT-IN/ST. These plasmids will transcribe greater than 7kb mRNA to express the Gag/Gag-PR or Gag/Gag-PR-RT-IN. To identify which of the Gag-Pro/ST or Gag-PR-RT-IN/ST still efficiently produce the high level of p24 antigen in the cell, one can test the different sized constructs against the parent plasmids, Gag-Pro or Gag-PR-RT-IN.

(c). Humanized Vpr-RT-IN gene to reduce homologous sequence

Expression of HIV structural proteins and enzymes, like those of the gag, reverse transcriptase, integrase, and envelope protein, is facilitated by a cis-acting element in the viral mRNA known as the rev-responsive element (RRE), and by the action of Rev since these viral mRNA contain the multiple inhibitory sequences (INS) (Malim et at (1989) Nature 338:254-257; Brighty et al (1994) PNAS 91:8314-8313).

A current hypothesis about the function of INS is that they are binding sites for cellular factors that contribute to instability. Recently, it has been demonstrated that substitutions of AT-rich regions in gag and env, mostly in the third-site position, without changing the amino acid sequence of the produced Gag and Env proteins, can result in efficient expression of Gag and Env in a Rev- and RRE-independent fashion (Schwartz et al. (1992) J. Virol. 66:7176-7182; Haas et al (1996) Current Biology 6:315-324). Recent studies suggested that the codon-usage effects are a major impediment to the efficient expression of HIV-1 genes, e.g., rev. Therefore, the creation of a synthetic coding sequence based on codons over-represented in highly expressed human genes overcomes a major limitation to the translational efficiency of HIV-1 Env. Moreover, this synthetic gene can result in efficient expression of Env protein in a Rev- and RRE-independent fashion (Haas et al (1996) Current Biology 6:315-324). Previous studies have indicated the profound bias in the codon usage of the Pol proteins (RT and IN) (Kypr et al (1987) Nature 327:20).

A synthetic Vpr-RT-IN gene based on optimal codon usage (humanized Vpr-RT-IN gene) will provide several advantages over the current Vpr-RT-IN gene. First, since about one-third of the sequence will change, the humanized Vpr-RT-IN plasmid should dramatically reduce the homologous recombination with the RT and IN part of packaging constructs. Second, the humanized synthetic gene can result in efficient expression of Vpr-RT-IN protein in a Rev- and RRE-independent fashion. Therefore, RRE sequence, which exists in both the packaging constuction and transfer vector, can be removed from the trans-enzyme plasmid. Fig. 27c shows the codon usage of highly expressed human genes (Haas et al (1997) Current Biology 6:315-324). Fig. 27b shows how to humanize Vpr, RT and IN genes by PCR.

Example 12: A Retrovirus-Based Vector That Enables Transduction of Non-dividing Cells

Lentiviruses such as HIV-1 can infect non-proliferating cells owing to the karyophilic properties of the lentiviral preintegration complex which governs recognition of the cell nuclear import machinery. Lentivirus-based vectors (HIV, FIV and EIAV) can transduce cell lines that are growth-arrested in culture, as well as macrophage, neuron, muscle and retina. However, the greatest concern about the clinical use of HIV-based vectors is linked to the possibility that the parental pathogenic virus might be reconstituted through genetic recombination.

Until now, retroviral vectors used in clinical trials have been primarily derived from oncoretroviruses such as the murine leukemia virus (MLV). These vectors can only transduce cells that divide shortly after infection because the MLV preintegration complex cannot migrate to the nucleus in the absence of mitosis. This considerably limits the use of MLV-based vectors for gene delivery into targets such as neurons, hepatocytes, myocytes and hematopoietic stem cells.

In this example, we disclose a novel approach that splits the structure of Gag/Gag-PR-RT-IN into two separate coding sequences: one that express the Gag and another that express the Gag-PR-RT-IN (Fig. 28). The production of the fixed ratio of Gag to Gag-PR-RT-IN will be regulated by the IRES, a cis-acting element. Since the Gag and Gag-PR-RT IN express from the two separate coding sequences, and Gag-PR-RT-IN is not required for the conserved stem-loop structure and downstream mRNA secondary structure, one can insert specific sequences into the C-terminus region of gag, via linkage to the nucleocapsid protein (NC) (Fig. 28). Since NC associates with the PIC, the modified NC containing a nuclear localization signal or other specific motif that facilitates nuclear transport will enable the PIC of an MLV-based vector to be transported into the nucleus in the absence of mitosis. Therefore, the modified MLV based vector packaging system will facilitate MLV-based vectors to transduce nondividing cells.

Recent studies suggested that the central DNA flap acts as a cis-determinant of HIV-1 DNA nuclear import. The location of the central flap has been precisely defined in the case of HIV-1. Central strand displacement starts at the first nucleotide following the cPPT sequence and stops 99 nucleotides downstream at the terminator 2 site of CTS. The

presence of a DNA flap at the center of the genome can probably be generalized to all lentiviruses. Thus, the reverse-transcribed HIV-1 genome bears a cis-acting determinant for its nuclear import. HIV-1 gene transfer vectors lacking the central DNA flap exhibit a strong nuclear import defect. This defect can be rescued by reinsertion of the DNA flap sequence into the transfer vector.

Thus provided is an improved MLV-based transfer vector that contains a DNA flat sequence derived from HIV-1. To facilitate initiating the central strand synthesis, the PPT sequence derived from MLV is inserted upstream of the cPPT sequence (Fig. 29). This novel design of the MLV-based vector improves the ability to transduce non-dividing cells.

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All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually. One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent. The methods and systsems described herein are exemplary and not intended as limitations on the scope of the invention. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present

invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Thus, additional embodiments are within the scope of the invention and within the following claims: